STIC-ILL

From: Baskar, Padmavathi
Sent: Tuesday, November 05, 2002 11:09 AM
To: STIC-ILL
Subject: 09/833017--DENTAL CARIES

PLEASE PROVIDE ME THE FOLLOWING ASAP. THANK YOU ALL

1. APPL.ENVIRON.MICROBIOL 1998, 64;2247-2255
2. APPL.ENVIRON.MICROBIOL 1999 65; 3710-3713
3. APPL.ENVIRON.MICROBIOL 1996, 62; 2994-2998
4. INFECT.IMMUN, 1983, 41; 722-27
5. INFECT.IMMUN, 1981, 32; 1295-1297
6. J.BACTERIOL, 2000, 182;1374-1382.

Padma Baskar
Art Unit 1645
Patent Examiner/Biotechnology
CM-1, 8E-13, BOX 8E-12
703-308-8886

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1998, p. 2247–2255
0099-2240/98/$04.00+0

Vol. 64, No. 6

Establishment of New Genetic Traits in a Microbial Biofilm Community

BJARKE B. CHRISTENSEN,[1] CLAUS STERNBERG,[1] JENS BO ANDERSEN,[1] LEO EBERL,[2] SØREN MØLLER,[3] MICHAEL GIVSKOV,[1] AND SØREN MOLIN[1]*

Department of Microbiology, The Technical University of Denmark, DK-2800 Lyngby,[1] and NovoNordisk A/S, DK-2880 Bagsværd,[3] Denmark, and Lehrstuhl für Mikrobiologie, Technische Universität München, D-80290 Munich, Germany[2]

Received 6 February 1998/Accepted 19 March 1998

Conjugational transfer of the TOL plasmid (pWWO) was analyzed in a flow chamber biofilm community engaged in benzyl alcohol degradation. The community consisted of three species, *Pseudomonas putida* RI, *Acinetobacter* sp. strain C6, and an unidentified isolate, D8. Only *P. putida* RI could act as a recipient for the TOL plasmid. Cells carrying a chromosomally integrated *lacI*^{q} gene and a *lacp-gfp*-tagged version of the TOL plasmid were introduced as donor strains in the biofilm community after its formation. The occurrence of plasmid-carrying cells was analyzed by viable-count-based enumeration of donors and transconjugants. Upon transfer of the plasmids to the recipient cells, expression of green fluorescence was activated as a result of zygotic induction of the *gfp* gene. This allowed a direct in situ identification of cells receiving the *gfp*-tagged version of the TOL plasmid. Our data suggest that the frequency of horizontal plasmid transfer was low, and growth (vertical transfer) of the recipient strain was the major cause of plasmid establishment in the biofilm community. Employment of scanning confocal laser microscopy on fixed biofilms, combined with simultaneous identification of *P. putida* cells and transconjugants by 16S rRNA hybridization and expression of green fluorescence, showed that transconjugants were always associated with noninfected *P. putida* RI recipient microcolonies. Pure colonies of transconjugants were never observed, indicating that proliferation of transconjugant cells preferentially took place on preexisting *P. putida* RI microcolonies in the biofilm.

Biological wastewater treatment, removal of organic compounds from contaminated soil, biogas reactors, etc., all involve processes based on the action of microbial communities. Increasing demands for improving the efficiencies of degradation in these systems have resulted in the need for a better understanding of the function and significance of the individual organisms in the community. One way to stimulate degradation of certain pollutants would be to introduce new genetic information into the microbial community. This process, termed bioaugmentation, involves the addition of new bacteria to the community, thus introducing new biodegradation pathways for metabolic conversion of the pollutants. However, enhancement of biodegradation often fails due to poor establishment and/or survival of the new strain in the environment (20, 25, 44). An alternative approach is to transfer the relevant genes on conjugative plasmids to indigenous organisms, from which the genes may spread further in the community (13, 15, 17, 18, 28, 32).

If plasmid genes (e.g., for resistance to heavy metals or antibiotics, for metabolic pathways, etc.) are acquired from foreign organisms and exchanged between members of the community, the capacity of a community to develop new microbial traits may be increased, making the community more responsive to environmental changes (19, 24, 40). However, little is known about the features which are important for the integration of new genetic traits by microbial communities.

Methods for determination of the organism composition and for tracing specific genes in microbial communities include the use of nucleic acid probes targeting specific DNA regions (for a review, see Sayler and Layton [34]), frequently by including a PCR step to amplify the region of interest (31, 39); in situ rRNA hybridization employing 16S or 23S rRNA probes targeting specific organisms (for a review, see Amann et al. [2]); or simple plating on selective plates.

During the last 5 to 10 years, several new techniques have been developed for more detailed analysis of complex microbial communities. The application of scanning confocal laser microscopy (SCLM) in combination with a number of fluorescent biomarkers has been used for analysis of structural features, such as the arrangement of cells, polymers, and channels in microbial environments (27, 46, 47). In situ hybridization with fluorescence-labeled 16S or 23S rRNA probes in combination with SCLM is a powerful approach for visualization of the spatial distribution of important group organisms in bacterial communities (29, 41).

Fluorescent markers may also be introduced by genetic engineering. The *gfp* gene encoding the green-fluorescent protein (Gfp) (7) and enhanced by Cormack et al. (10) has been very useful as a reporter for studies of gene expression in single cells. For example, fusions between the relevant promoter and the *gfp* gene have been used to monitor subcellular protein localization during sporulation in *Bacillus subtilis* (42) and to analyze mycobacterial interactions with macrophages (14). Gfp has also been used as a reporter to monitor the kinetics of TOL plasmid transfer between bacteria growing on agar surfaces (8) as well as to track individual cells in an activated-sludge community (16).

In the present work, we have investigated the establishment of the TOL plasmid in a biofilm community growing on benzyl alcohol as the sole carbon and energy source. A consortium of bacteria, originally isolated from a creosote-polluted aquifer, was previously used in a waste gas biofilter for the degradation of toluene (29). From this complex consortium of bacteria, a

* Corresponding author. Mailing address: Department of Microbiology, Building 301, The Technical University of Denmark, DK-2800 Lyngby, Denmark. Phone: 45 45 25 25 13. Fax: 45 45 88 73 28. E-mail: sm@im.dtu.dk.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli K-12		
CC118λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA thi-1 rpsE rpoB argE*(Am) *recA* λpir lysogen	21
HB101	*E. coli* K-12/B hybrid; Sm^r *recA thi pro leu hsdRM*$^+$	4
P. putida		
RI (SMO116)	Natural isolate; γ subgroup of class *Proteobacteria*[a]	29
JB154	SMO116 Rif^r	This work
JB156	SMO116 Nal^r	This work
KT2440	*hsdR*/TOL	K. Timmis
KT2442	*hsdR* Rif^r	K. Timmis
SM1443	KT2442 *lacI*^{q}; *npt* removed by site-specific recombination	This work
BBC443	SM1443/TOL*gfp*mut3b	This work
BBC458	JB156/TOL*gfp*mut3b	This work
BBC516	JB154/TOL*gfp*mut3b	This work
Acinetobacter sp. strain C6 (SMO112)	Natural isolate; γ subclass of class *Proteobacteria*[b]	29
Unknown genus D8 (SMO125)	Natural isolate; β subclass of class *Proteobacteria*[c]	29
Plasmids		
RK600	Cm^r ColE1 *oriV* RP4 *oriT*; helper plasmid in triparental matings	22
pUC18Not	Same as pUC18 (49) but with polylinker flanked with *Not*I sites	21
pUTKm	Ap^r Km^r; transposon delivery vector for mini-Tn*5* Km	11
pCK242	mini-Tn*5* transposon delivery vector; *npt* gene flanked by *res* sites	23
pJBA27	*Sph*I-*Hin*dIII *gfp*mut3b fragment cloned downstream of $P_{A1/04/03}$	3
pJBA28	*Not*I fragment $P_{A1/04/03}$::*gfp*mut3b from pJBA27 cloned into pUT mini-Tn*5* Km	3
pSM1431	*lacI*^{q} fragment (38) cloned into pUC18Not (*Sca-Sal*I)	This work
pSM1435	*Not*I fragment (*lacI*^{q}) of pSM1431 inserted into pUTKm	This work
TOL	Self-transmissible approximately 117-kb plasmid; from *P. putida* mt-2	43
TOL*gfp*mut3b	Integration of $P_{A1/04/03}$::*gfp*mut3b from pJBA28 into TOL	This work

[a] From reference 29.
[b] Based on 16S rRNA sequence.
[c] Based on hybridization with group-specific probes.

model community consisting of three species (*Pseudomonas putida* RI; a strain of *Acinetobacter* sp.; and a strain, D8, tentatively associated with the β subgroup of *Proteobacteria* strains), isolated as individual clones from the original waste gas biofilter, was defined for our biofilm investigations. In situ rRNA hybridization was used to identify the three species present in the community, and an approach based on zygotic induction of the Gfp protein developed to study plasmid transfer directly in the community (8) was employed in order to localize conjugational activities.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids and their relevant characteristics are listed in Table 1. The strains were grown in Luria-Bertani (LB) broth (containing 10 g of tryptone, 5 g of yeast extract, and 4 g of NaCl). When required, antibiotics were added at final concentrations of 50 μg/ml for rifampin and nalidixic acid and 10 μg/ml for kanamycin.

Mutants of the natural isolate *P. putida* RI, resistant to either nalidixic acid or rifampin, were isolated as spontaneous mutants on LB broth plates containing concentrations of 100 μg of nalidixic acid or rifampin per ml, respectively.

A modified version of the pUT vector (12), comprising a mini-Tn*5* transposon with RP4 resolvase sites flanking the *npt* gene responsible for kanamycin resistance (pCK242) (23), was used for insertion of the *lacI*^{q} gene (38) into the chromosome of KT2442. Triparental mating among a donor strain (carrying the *lacI*^{q} delivery plasmid pSM1435), the helper strain HB101(RK600), and the recipient *P. putida* KT2442 resulted in KT2442 derivatives conferring kanamycin resistance with the *lacI*^{q} gene inserted in the chromosome. Subsequently, the *npt* gene was deleted as described by Christensen et al. (8). One such kanamycin-sensitive clone was picked and designated SM1443.

The gene encoding the green fluorescent protein Gfpmut3b was obtained from Cormack et al. (10). The *gfp*mut3b gene was PCR amplified as a 0.7-kb *Sph*I-*Hin*dIII fragment. When introducing a *Sph*I site in the start codon of *gfp*mut3b the sequence was changed in the PCR such that the Gfpmut3b protein contained an arginine instead of a serine residue at position 2.

The *gfp*mut3b fragment was cloned downstream of the promoter $P_{A1/04/03}$ (6), at an optimal distance from a synthetic ribosome binding site (RBSII, from plasmid pQE70; Qiagen GmbH, Germany), and upstream of a region with two strong transcriptional terminators, T_0 (from phage lambda) and T_1 (from the *rrnB* operon of *Escherichia coli*), and with translational stop codons in all three reading frames. The *Not*I fragment from the resulting plasmid (pJBA27), containing RBSII, *gfp*mut3b, the translational stop codons, and the transcriptional terminators, was inserted into the *Not*I site of pUTKm, resulting in the transposon delivery vector pJBA28, containing a cassette with $P_{A1/04/03}$::*gfp*mut3b and the *npt* gene.

Insertion of the $P_{A1/04/03}$::*gfp*mut3b cassette into the TOL plasmid was performed in two steps. First, a triparental mating was performed, in which the helper plasmid RK600 was used to mobilize the delivery plasmid pJBA28 from the donor strain, CC118λ*pir*, into the recipient strain, *P. putida* KT2440. Selection on AB-minimal plates (9) containing 10 mM citrate and 50 μg of kanamycin/ml resulted in KT2440 derivatives carrying the $P_{A1/04/03}$::*gfp*mut3b cassette inserted either in the chromosome or in the TOL plasmid. Clones with the cassette integrated in the TOL plasmid were selected from a second round of conjugation. Colonies from the first-round selective plates (>1,000 on one plate) were suspended in 1 ml of 0.9% NaCl. The suspended cells were then mated with the kanamycin-sensitive recipient, SM1443. Selection on plates containing 50 μg of kanamycin/ml and 50 μg of rifampin/ml resulted in SM1443 Km^r derivatives carrying the TOL plasmid with the $P_{A1/04/03}$::*gfp*mut3b cassette inserted at random positions. One clone (designated BBC443) was chosen for subsequent experiments based on the following criteria: it was able to grow on AB-minimal plates supplemented with either 5 mM benzyl alcohol or 5 mM sodium benzoate as the sole carbon and energy source; the colonies were green fluorescent upon addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside); and finally, the conjugation frequency on plates of the *gfp*-tagged plasmid was similar to that of the wild-type TOL plasmid.

Cultivation of biofilms. The biofilm model community comprised the following organisms: *P. putida* RI JB156, which is resistant to nalidixic acid, or JB154, which is resistant to rifampin; *Acinetobacter* sp. strain C6; and an unidentified

strain, isolate D8, of the β subgroup of the class *Proteobacteria* (Table 1). All of the strains are able to mineralize toluene and benzyl alcohol (30).

Biofilms were cultivated as mixtures of the three species in rectangular two- or four-channel flow cells (47) with individual channel dimensions of 1 by 4 by 40 mm supplied with a flow of FAB substrate [1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM Fe-EDTA [catalog no. E6760; Sigma, St. Louis, Mo.), 0.15 mM $(NH_4)SO_4$, 0.33 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 0.5 mM NaCl]. Benzyl alcohol (Merck KGaA, Darmstadt, Germany) at a concentration of 0.5 mM was used as the sole carbon source.

Flow chamber experiments. The flow system was assembled with autoclaved silicone tubing. Complete sterilization was performed by pumping a solution of 0.5% sodium hypochlorite into the system and leaving it overnight. The following day approximately 0.2 liters of sterile water per flow chamber was flushed through the system before the medium was pumped in.

During biofilm growth, the medium was pumped through the flow cells at a rate of 0.2 mm/s with a peristaltic pump (model 205S; Watson-Marlow Inc., Wilmington, Mass.). The flow cells were inoculated with mixtures of cultures of the three strains pregrown for 2 days in LB medium in the ratio 1:10:5 for *P. putida*, *Acinetobacter*, and isolate D8, respectively. The mixtures were sonicated with a Branson sonifier (Branson Ultrasonics Corp., Danbury, Conn.) for 1 min at output control 3 and duty cycle 40%, and 0.25 ml of the mixed culture was injected into each channel.

Sequencing of 16S rRNA. Sequencing of 16S rRNA was performed with an automatic 373A DNA sequencer (Applied Biosystems, Foster City, Calif.) directly on PCR products generated from chromosomal DNA extracts according to the manufacturer's recommendations. The following primers were used (in the 5′→3′ direction): 11F, GTTTGATC(A/C)TGGCTCAGATTG; 344R, CCCCA CTGCTGCCTCCCGT; 515R, GTATTACCGCGGC(G/T)GCTGGCAC; 922R, GCTTGTGCGGGCCCCCGTC; 1101R, GACAAGGGTTGCGCTCGTT; 1389R, GTGACGGGCGGTGTGTACAAG; and 1465R, CCCCAGTCATGAATCATA AAGTGGT. Initial phylogenetic analysis of the sequenced rRNAs was obtained from on-line services of the Ribosomal Database Project (SIMILARITY_RANK [26]). In addition, potential signature sequences were identified as described by Woese (45), allowing differentiation between the major groups of the class *Proteobacteria*.

Oligonucleotide probes. For 16S rRNA hybridization, the probes EUB338 (specific for the domain *Bacteria* [1]) and PP986 (specific for the *P. putida* subgroup A [29]) were used. Based on the sequence information obtained, specific rRNA probes for *Acinetobacter* sp. strain C6 and isolate D8 were designed. The specificities of Acn449 and D8_647 were tested against published sequences with the CHECK_PROBE program from the Ribosomal Database Project (26). All probes were tested against the organisms of the community and found to be specific for their respective target organisms (data not shown). Oligonucleotide probes labeled with fluorescein isothiocyanate or the indocarbocyanine fluorescent dyes CY3 and CY5 were purchased from Hobolth DNA Syntese (Hillerød, Denmark).

Embedding of hydrated biofilm samples. Embedding was performed as a nondestructive method to maintain a fixed biofilm in its 3-dimensional native hydrated state and at the same time to allow easy handling of the fixed biofilm. Throughout the embedding procedure, pumping of solutions through the flow cells was performed at 0.8 mm/s. Biofilms were fixed in freshly made 3% paraformaldehyde solution (33) by pumping the solution through the flow channels with attached biofilms. To ensure the complete fixation of all cells in the biofilms, the solution was retained in the channels for 1 h at room temperature before the biofilms were washed by flowthrough of phosphate-buffered saline for 5 min. Embedding was performed by introducing a solution of 1 ml of 20% acrylamide (200:1 acrylamide-bisacrylamide) mixed with 8 μl of *N,N,N′,N′*-tetramethylethylenediamine (Kodak International Biotechnologies Inc., New Haven, Conn.), and immediately prior to inoculation, 20 μl of ammonium persulfate (Kodak International Biotechnologies Inc.) was also added. Approximately 0.5 ml of the solution was pumped into the channel, where it solidified within 2 min after addition of the ammonium persulfate.

Hybridization of embedded biofilm cells. After fixation and embedding, the polyacrylamide block with biofilm was placed on a 6-well hybridization slide (Novakemi AB, Enskede, Sweden) and equilibrated for 15 min with hybridization buffer containing 30% formamide at 37°C. Then 30 μl of hybridization mixture (30% formamide, 0.9 M NaCl, 100 mM Tris [pH 7.2], 0.1% sodium dodecyl sulfate) containing 75 ng of probe was added to each hybridization well. Cells were incubated with hybridization solution for 3 h at 37°C in a moisture chamber. For washing, 50 μl of each washing solution was added to each well as follows. First, the acrylamide blocks were washed in solution I (30% formamide, 0.9 M NaCl, 100 mM Tris [pH 7.2], 0.1% sodium dodecyl sulfate) for 40 min at 37°C, then they were washed for 40 min in washing solution II (0.1 M Tris [pH 7.2], 0.9 M NaCl) at 37°C, and finally, they were rinsed two times in 50 μl of distilled water. The acrylamide blocks were mounted in 2× SlowFade phosphate-buffered saline-based antifade solution (Molecular Probes, Eugene, Oreg.).

Microscopy and image analysis. All microscopic observation and image acquisition was performed on a TCS4D confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with three detectors and filter sets for simultaneous monitoring of fluorescein isothiocyanate/green-fluorescent protein and the indocarbocyanine dyes CY3 and CY5.

Multichannel simulated fluorescence projection (SFP; a shadow projection) images and vertical cross sections through the biofilms were generated with the IMARIS (Bitplane AG, Zürich, Switzerland) software package running on an Indigo 2 (Silicon Graphics, Mountain View, Calif.) workstation. The images were further processed for display with Photoshop software (Adobe, Mountain View, Calif.).

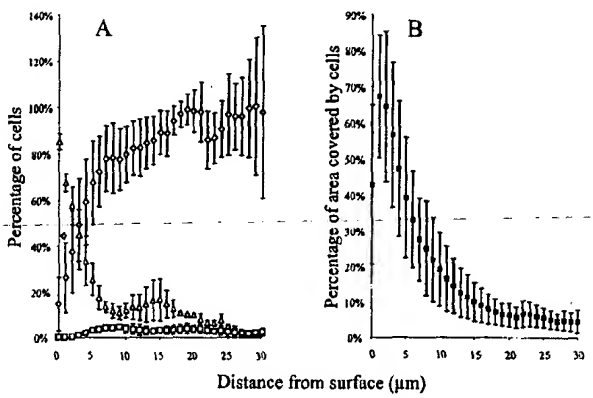

FIG. 1. Quantitative analysis showing the spatial distribution of the different organisms of the mixed-culture biofilm sampled at day 7. (A) Vertical profile through the biofilm showing percentage of *Acinetobacter* sp. strain C6 (△), *P. putida* RI (◇), and isolate D8 (□) relative to the total number of cells targeted with the general eubacterial 16S rRNA probe (EUB338 [1]). Each profile is an average of three images (each consisting of 31 optical sections) captured at three random locations in the biofilm. (B) Relative area covered by cells in each section, taken as an average over six images. Standard deviations are indicated by error bars.

The spatial distribution of organisms was estimated by measuring the area covered by hybridization signal (represented by different colors) in series of two-channel optical sections by using simple thresholding. One channel represented the cells targeted with the general eubacterial probe EUB338 (1), and the other channel represented the cells hybridized with a species-specific probe. Before thresholding was done, the images were preprocessed with IMARIS: background was removed by a lowpass filtering and background subtraction, and a final correction for loss of intensity from deeper layers was performed with the emission attenuation algorithm of IMARIS. Thresholding was performed on the processed images with the HIPS image analysis package.

Nucleotide sequence accession number. The sequence reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Y11464 (*Acinetobacter* sp. strain C6) and Y11465 (isolate D8).

RESULTS AND DISCUSSION

The model bacterial community. Flow chamber biofilms were established by mixing three strains which had been precultured separately for 2 days at 30°C in LB media prior to inoculation. Benzyl alcohol was added as the sole carbon and energy source for growth of the community. A quantitative analysis of the distribution of the different community members at different depths of a 7-day-old biofilm was done by probing with fluorescent 16S ribosomal DNA probes. Probes targeting the three selected species, *P. putida*, *Acinetobacter* sp. strain C6, and isolate D8, were used for identification. The analysis (Fig. 1A) revealed that *P. putida* RI was the most common species in the upper layers, whereas *Acinetobacter* dominated the layers near the substratum, where most of the overall biomass was observed (Fig. 1B). Isolate D8 was not a dominant community member at any depth of the biofilm.

Introduction of genes encoding the TOL biodegradation pathway into the flow chamber community. The three species comprising the microbial community are able to utilize benzyl alcohol as their sole carbon and energy source. Based on DNA hybridization and investigations of substrate profiles and metabolite formation, we have previously concluded that all the strains seem to harbor degradation pathways similar to that of the *P. putida* plasmid TOL (pWWO) (48). However, despite

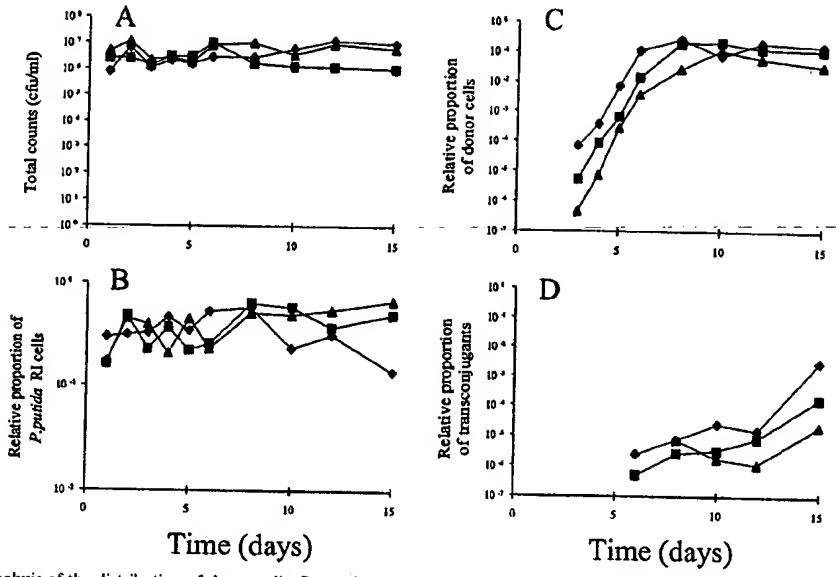

FIG. 2. Time course analysis of the distribution of donor cells, *P. putida* RI, and transconjugants relative to the total number of cells collected from flow channel effluents. The donor (*P. putida* RI/TOL*gfp*mut3b) was introduced at day 2 in three different densities: $5 \cdot 10^4$ (▲), $5 \cdot 10^5$ (■), and $5 \cdot 10^6$ (◆) CFU/ml. Total counts (A) were enumerated on pure LB broth plates. The *P. putida* RI cells (Nal^r) (B), donor cells (Rif^r) (C), and transconjugants (Nal^r Km^r) (D) were enumerated on LB broth plates containing the appropriate antibiotics, and the numbers were taken relative to the total counts. Each data point is the average of two independent experiments.

the similarities, the three pathways are probably not identical (30).

To analyze the establishment of a new metabolic pathway in the microbial community, the TOL plasmid was chosen. The TOL plasmid derivative TOL*gfp*mut3b (Table 1), which carries a fluorescent reporter tag and a kanamycin resistance marker gene, was used for this investigation. When grown in batch culture with benzyl alcohol as the sole carbon source the doubling time of *P. putida* RI was approximately 110 min (not shown). Introduction of the TOL plasmid into this strain by conjugation decreased the doubling time to approximately 80 min. The TOL plasmid had no significant effect on host cell generation times in media supplemented with carbon sources unrelated to the TOL pathway.

Establishment of the TOL plasmid degradation pathway in the biofilm community could occur through colonization of the biofilm by the plasmid-carrying donor strain, through plasmid transfer to the indigenous bacteria, or both. In the experiment, these possibilities could be monitored independently, and the experimental results are presented accordingly.

The population size of the incoming donor strain—a rifampin-resistant derivative of *P. putida* RI carrying the plasmid TOL*gfp*mut3b (BBC516)—was determined as rifampin- and kanamycin-resistant viable counts present in the effluent from the flow chamber. These determinations represent good estimates of the total biofilm populations, as indicated by control experiments in which entire biofilm communities have been analyzed and compared with effluents (not shown). Transfer of the TOL plasmid to the indigenous nalidixic acid-resistant strain of *P. putida* RI was estimated from cell counts on selective plates supplemented with nalidixic acid and kanamycin. Reversal of the antibiotic marker phenotypes in the incoming and indigenous strains of *P. putida* did not influence the outcome of the experiment (not shown).

The donor strain was introduced 2 days after the initial colonization of the model community. Three different concentrations of an overnight culture ($5 \cdot 10^4$, $5 \cdot 10^5$, or $5 \cdot 10^6$ CFU/ml) were injected into separate flow channels. Total cell counts in the effluents, as well as relative numbers of the indigenous *P. putida* RI cells, were marginally affected by the introduction of the *P. putida* RI cells carrying the TOL plasmid (Fig. 2A and B). However, as shown in Fig. 2C, the relative proportion of incoming *P. putida* RI cells started to increase exponentially immediately after their introduction. Independent of their abundance at the start of the experiment, they reached a steady-state level of approximately 10% of the total count 6 to 7 days after their introduction. Despite their apparently more effective degradation pathway (faster growth) and their rapid rate of establishment during the first few days, *P. putida* RI cells carrying the TOL plasmid did not outcompete the indigenous *P. putida* RI cells (Fig. 2C), even over a period of 40 days (data not shown).

Mating experiments performed on an agar surface showed that the TOL*gfp*mut3b plasmid could only be transferred to *P. putida* RI. When the donor cells (*P. putida* RI/TOL*gfp*mut3b) and the isogenic recipient cells (*P. putida* RI) were mixed in equal numbers on an agar surface, approximately 20% of the recipients hosted the plasmid after 24 h. Based on these results, it was surprising to see that plasmid transfer occurring in the biofilm community was almost negligible during the first several days and remained low throughout the experiment (Fig. 2D).

In conclusion, the data presented in Fig. 2 show (i) that establishment of the incoming donor strain in the biofilm was possible; (ii) that the TOL plasmid was established mainly by growth of the incoming donor cells (vertical transfer), possibly facilitated by the growth advantage mediated by the TOL degradation pathway; and (iii) that the TOL plasmid was transferred at a low but measurable rate to recipient cells in the community.

In a separate experiment, the influence of the strain background of the incoming organism was investigated. The strain *P. putida* KT2442 (Rif^r) carrying the TOL*gfp*mut3b plasmid is not isogenic with *P. putida* RI, although the 16S rRNA se-

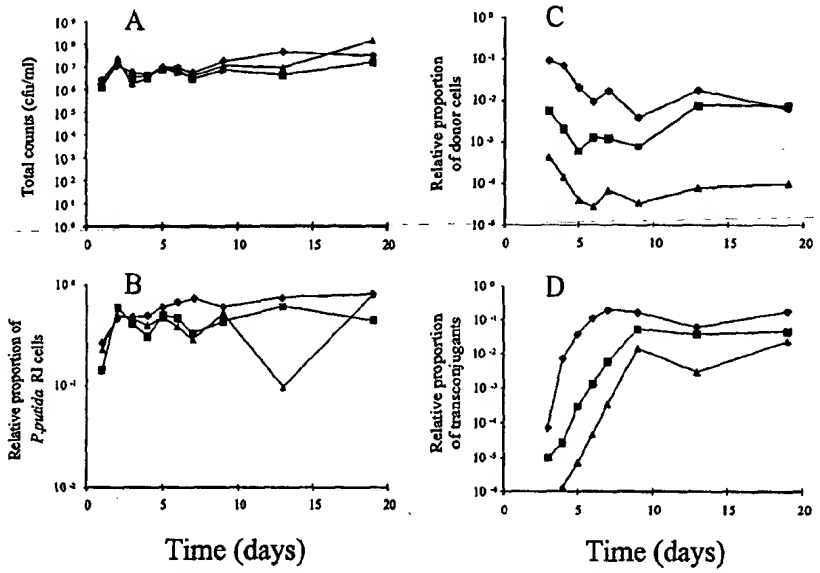

FIG. 3. Time course analysis of the distribution of donor cells, *P. putida* RI, and transconjugants relative to the total number of cells collected from flow channel effluents. The donor (*P. putida* KT2442/TOL*gfp*mut3b) was introduced at day 2 in three different concentrations: $5 \cdot 10^6$ (▲), $5 \cdot 10^7$ (■), and $5 \cdot 10^8$ (◆) CFU/ml. Total counts (A) were enumerated on pure LB broth plates. The *P. putida* RI cells (Nal^r) (B), donor cells (Rif^r) (C), and transconjugants (Nal^r Km^r) (D) were enumerated on LB broth plates containing the appropriate antibiotics, and the numbers were taken relative to the total counts. Each data point is the average of two independent experiments.

quences of the two strains only differ by a few bases (unpublished data). In addition, strain KT2442 grows in batch culture with benzyl alcohol as the sole carbon source with a doubling time of approximately 80 min, which is significantly faster than that measured for cultures of *P. putida* RI. Furthermore, when *P. putida* KT2442 (Rif^r) carrying the TOL*gfp*mut3b plasmid was cocolonized with the other three species, the strain constituted more than 90% of the total cell counts only a few days after colonization (data not shown), indicating that the strain is a good colonizer and competitor when introduced together with the three community strains.

In the following experiment, presented in Fig. 3, the impact of the donor cell background on the efficiency of establishment in the biofilm was investigated. The donor strain (KT2442/TOL*gfp*mut3b) was introduced 2 days after the initial colonization of the microbial flow chamber community. The incoming donor was introduced into separate flow chambers in three different concentrations ($5 \cdot 10^6$, $5 \cdot 10^7$, or $5 \cdot 10^8$ CFU/ml) of an overnight culture. Time course analysis of the population profile of cells collected from the effluents showed that neither the total cell count nor the relative proportion of *P. putida* RI cells was significantly affected by the introduction of the new strain (Fig. 3A and B).

Figure 3C shows that the relative number of donor cells collected from the flow channel effluents during the first few days after their introduction reflected the differences in the inoculation concentrations. Moreover, there was an initial phase of washing out of this strain, in contrast to what was observed for incoming *P. putida* RI cells (Fig. 2C). It should also be noted that KT2442, when introduced at a concentration of $5 \cdot 10^6$ CFU/ml, was established in the community at a 1,000-fold-lower level than that observed for the RI strain. Thus, when KT2442 harboring the TOL plasmid was introduced after initial colonization of the three community species it did not establish itself very effectively, and therefore the apparent growth advantage over the indigenous RI strain, as observed in batch cultures of suspended cells, was not sufficient to compete effectively in the biofilm community.

The relative proportion of transconjugants produced upon transfer of the TOL*gfp*mut3b plasmid to the indigenous *P. putida* RI cells increased rapidly until it reached a steady-state level of approximately 10% of the total population (Fig. 3D). The accumulation of transconjugants (*P. putida* RI/TOL*gfp*mut3b Nal^r) in this experiment and the accumulation of the introduced donor cells (*P. putida* RI/TOL*gfp*mut3b Nal^r) in the previous experiment show similar kinetics (compare Fig. 2C and 3D). This suggests that once *P. putida* RI cells carrying the TOL plasmid are formed or introduced in the biofilm, they grow and become established independently of the way in which they arise in the population. The results also suggest that the dominant mode of establishment of the TOL plasmid in the present community is through the rapid growth of cells representing an optimal host-plasmid combination; actual plasmid transfer plays a quantitatively minor role, which only becomes significant in situations where the donor strain cannot establish itself directly. This suggests that despite the difficulty with which new organisms may become established in already existing microbial communities, new genetic information can be introduced quite effectively when located on mobile elements, even if the actual transfer rates are low.

Similar observations have been made in more complex ecosystems: in soil microcosms (5, 13) and in freshwater flow-through systems (17, 18), strains carrying catabolic plasmids were found to be outcompeted shortly after their introduction, whereas the plasmids were transferred and established in a number of different indigenous community strains.

Vertical TOL plasmid transfer is dependent on the existing pool of *P. putida* RI cells in the biofilm. In the experiments described so far the biofilm community has been kept constant, i.e., the relative proportion of recipient *P. putida* RI cells has been high, constituting approximately half of the total population (Fig. 2 and 3). The influence of recipient concentration

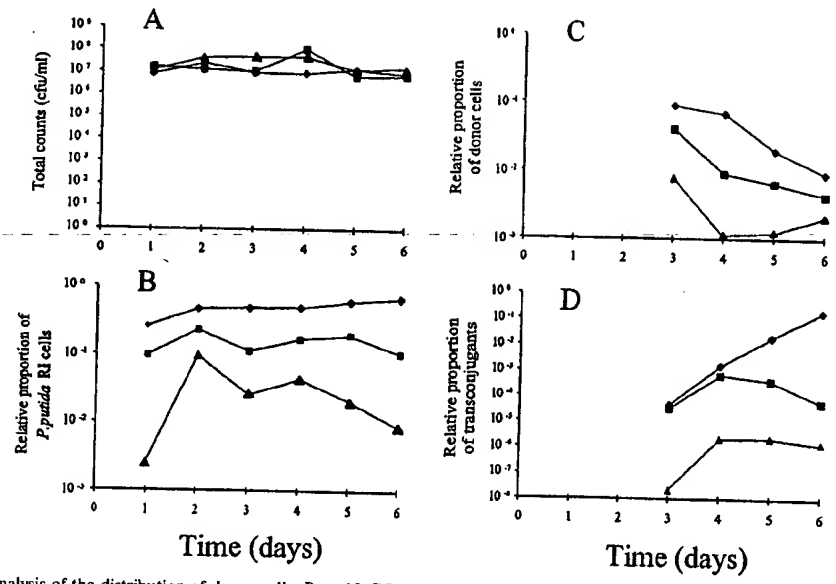

FIG. 4. Time course analysis of the distribution of donor cells, *P. putida* RI, and transconjugants relative to the total number of cells collected from flow channel effluents. Without changing the inoculation concentration of the two other isolates in the model community, *P. putida* RI was introduced in three different concentrations of 10^5 (▲), 10^7 (■), and 10^8 (◆) CFU/ml. Donor cells (*P. putida* KT2442/TOL*gfp*mut3b) ($5 \cdot 10^8$ CFU/ml) were introduced at day 2. Total counts (A) were enumerated on pure LB broth plates. The Nal^r *P. putida* RI cells (B), Rif^r donor cells (C), and Nal^r Km^r transconjugants (D) were enumerated on LB broth plates containing the appropriate antibiotics, and the numbers were taken relative to the total counts. Each point is the average of two individual experiments.

in the flow chambers on the rate of plasmid transfer and replication was investigated subsequently. Three channels were cultivated with the standard mixed-culture inoculum in the first channel (as in the experiments described above), and in the other two channels we reduced the number of inoculated *P. putida* RI cells 10 and 1,000 times, respectively, relative to the number of *P. putida* RI cells introduced in the first channel.

Figure 4B shows that, when introduced in low numbers, *P. putida* RI cells grew rapidly during the first 2 days after colonization, followed by a slow decline. In all three channels, donor cells of the *P. putida* strain KT2442 harboring the TOL plasmid described above were introduced at day 2 at a concentration of 10^8 CFU/ml. Figure 4C shows that the relative proportion of donor cells remaining in the biofilm decreased approximately 1 order of magnitude over the next 3 to 4 days in all channels, in agreement with the results shown in Fig. 3C, confirming that this strain of *P. putida* does not become established easily in the community. Accumulation of transconjugants occurred during the first 2 days (Fig. 4D), but only in the channel with the highest concentration of indigenous *P. putida* RI cells did accumulation continue. It is striking that even small decreases in the relative numbers of *P. putida* RI cells in the community eventually led to a cessation of transconjugant accumulation. Thus, the size of the preexisting pool of *P. putida* RI cells in the biofilm community significantly influenced transconjugant establishment and the introduction of new genetic traits.

In situ visualization of transconjugants in the biofilm community. Tagging of the TOL plasmid with a *gfp* gene (encoding the green fluorescent protein Gfpmut3b) fused to the strong *lac* promoter $P_{A1/04/03}$ (see Materials and Methods) allowed us to monitor the in situ establishment of the plasmid within the biofilm. To specifically follow the occurrence and growth of transconjugants, we further inserted the *lacI* gene in the chromosome of the donor strain, *P. putida* KT2442, resulting in repression of *gfp* expression from the plasmid. Thus, expression of the *gfp* gene will be induced upon transfer of the TOL plasmid to a recipient in the community (*P. putida* RI) not harboring the *lacI* gene (zygotic induction of fluorescence).

In an experiment where donor cells were introduced at a concentration of 10^6 CFU/ml, regions with high densities of transconjugants were observed. One such "high-density zone" was observed for 2 days. Five days after introduction of the donor a strongly green-fluorescent microcolony was observed (Fig. 5A), and other fluorescent microcolonies were found downstream but not upstream of this microcolony. In the following days a progressively increasing number of fluorescent microcolonies were detected in this hot-spot region (Fig. 5B).

Based on the results obtained here and described above, we suggest that in the first microcolony shown in Fig. 5A the TOL plasmid initially transferred from a donor cell (*P. putida* KT2442) to a recipient cell (*P. putida* RI). The growth advantage of the resulting transconjugant cell allowed the transconjugant cell and its progeny to grow quickly and consequently to produce a cluster of fluorescent cells. Subsequently, some of the cells detached and were carried by the substrate flow further downstream, where they again became established, forming new clusters of transconjugants. According to this interpretation, each "colony" of transconjugants in a flow channel arose from a single horizontal plasmid transfer event followed by effective proliferation of the progeny cells.

The more precise organization of the transconjugants relative to the indigenous recipient cells of *P. putida* RI and the other members of the community was investigated 8 days after introduction of 10^8 CFU of donor cells of *P. putida* KT2442/ml, carrying the *gfp*-tagged TOL plasmid. The biofilm was fixed and embedded, and in situ hybridization was performed in combination with SCLM. By using the probe PP986 targeting *P. putida* cells, the spatial distribution of transconjugant (fluorescent) cells and noninfected recipient cells could be visualized. In addition, the probe ACN449 was used to target *Acinetobacter*, the other dominant member of the community.

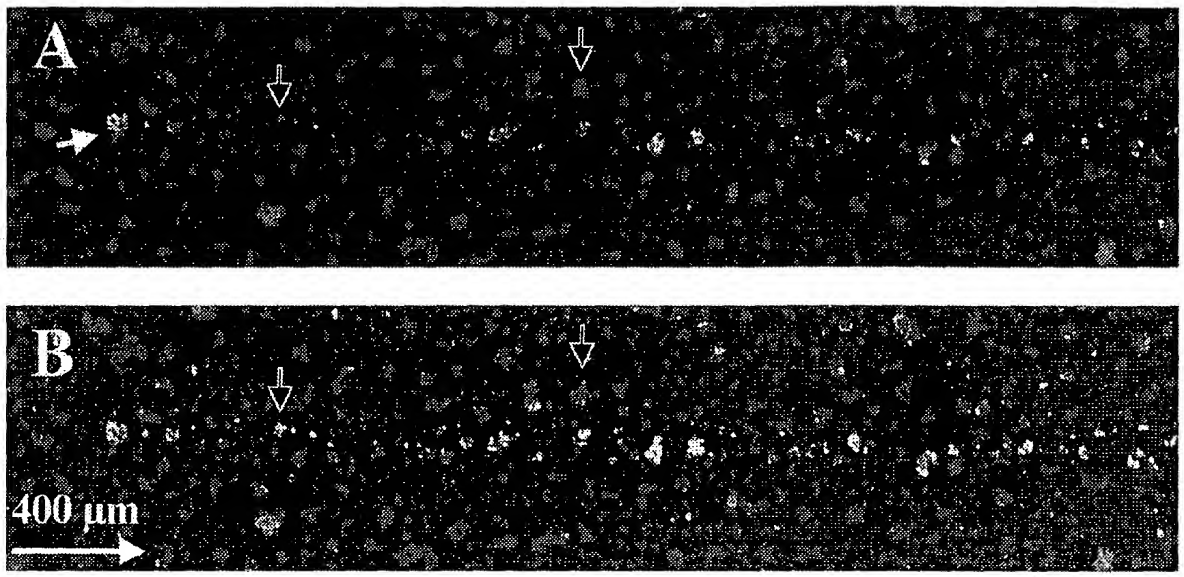

FIG. 5. On-line monitoring of transconjugant proliferation on microcolonies in the direction of flow at days 5 and 6 after donor introduction. The white patches are regions with strong green-fluorescent signal (microcolonies with transconjugants), and the gray regions are weak autofluorescent signals emitted from *P. putida* cells. This signal is easy to distinguish from the strong green-fluorescent signal emitted from cells expressing Gfp and could be used to visualize the location of noninfected microcolonies. On day 5 (A) a strongly green-fluorescent microcolony (solid arrow) was observed and other green-fluorescent microcolonies were located in a region straight downstream from this colony, but not upstream. On day 6 (B) more green-fluorescent colonies were observed. The scale bar also indicates the direction of flow. Open arrows indicate examples of microcolonies which had been infected with transconjugants from day 5 to day 6.

Obviously, the donor and recipient *P. putida* strains cannot be distinguished with the specific 16S ribosomal DNA hybridization probe used here. However, due to the poor establishment of the *P. putida* KT2442 donor cells relative to the *P. putida* RI recipient cells (compare Fig. 3C with B), most *P. putida* cells identified by the PP986 probe were assumed to be *P. putida* RI cells. Figure 6A shows a representative region of the biofilm.

Similar to the data presented in Fig. 1A, *Acinetobacter* was preferentially located in the substratum and *P. putida* RI cells were located in the upper layers, organized in clusters (microcolonies) sticking up from the surface. With few exceptions, the *P. putida* RI microcolonies were covered with green-fluorescent transconjugant cells.

A magnification of a microcolony is shown in Fig. 6B. A vertical cross section of the colony further shows how the transconjugants appear in layers of cells covering the colony. Inspection of a number of images like the one presented in Fig. 6 showed that transconjugant cells only very rarely became established as new microcolonies. Instead, they were observed to be preferentially on top of already established microcolonies of recipient cells. This supports the results shown in Fig. 5, where it was observed that the green-fluorescent clusters always showed up on existing microcolonies. Thus, the dominant mode of establishment of cells harboring new genetic information seems to be colonization of existing recipient microcolonies in the flow chambers. This would explain why accumulation of transconjugants in the biofilm is strongly dependent on the relative concentration of recipient *P. putida* RI cells (as indicated in Fig. 4).

Although the transconjugant and recipient cells seemed to be extremely tightly associated in the microcolonies, horizontal transfer of the TOL plasmid through the entire recipient colony was never observed. This observation is analogous to our previous finding that TOL plasmid transfer between isogenic *P. putida* KT2442 donor and recipient colonies growing on an agar surface only occurred in a narrow border zone between the two colonies (8).

A number of explanations may account for these observations. Previous studies have shown that TOL plasmid transfer is strongly dependent on the physiological state of the bacteria (36, 37). If the *P. putida* RI microcolonies contained a steep substrate gradient from the surface to the inner parts, only cells at the surface of the microcolony would be exposed to nutrient concentrations sufficiently high to allow plasmid transfer.

Another explanation could be that although the TOL plasmid has been reported to be naturally derepressed for pilus synthesis (5), this might only be true for cells grown under optimal conditions in well-defined environments. In more complex environments, where cells are organized in dense microbial communities, newly formed transconjugants may be exposed to a number of environmental signals, which could cause repression of pilus synthesis and consequently suppress further conjugation.

It is also possible that the recipient cells in a colony and the newly formed transconjugant cells on the surface of the colony will grow and develop as separate cell lines due to small differences in their phenotypes. Studies of colonies growing on agar surfaces have shown that even very small changes (a single mutation) in a cell may give rise to the formation of colony sectors caused by individual cell lines growing out from the center in separate zones (8, 35).

Finally, it is possible that the Gfp protein does not fold correctly due to a low oxygen tension in the middle of a colony—a possibility that was tested as follows. The biofilm in one flow chamber was disintegrated, and it was found that the relative proportion of green-fluorescent cells constituted approximately 10% of the total population when counted directly under the microscope, a number which correlated well with the proportion of kanamycin- and nalidixic-resistant cells (i.e.,

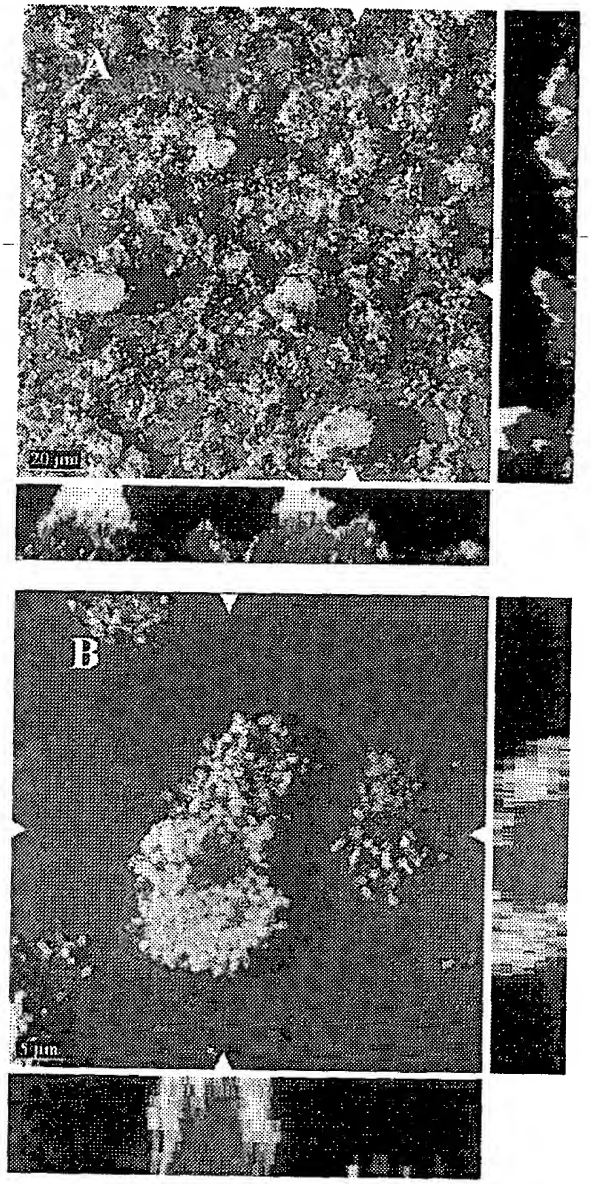

FIG. 6. (A) Spatial distribution of green-fluorescent transconjugants (green or yellow) relative to noninfected *P. putida* RI cells and *Acinetobacter* sp. strain C6 in a biofilm analyzed 8 days after introduction of donor cells. The organisms *P. putida* (red) and *Acinetobacter* sp. strain C6 (purple) were identified by hybridization. After hybridization, green-fluorescent transconjugants appear as either yellow or green, depending on the ratio between the green Gfp signal and the red hybridization signal. The x-y plot is presented as a SFP, where long shadows indicate a large and/or high microcolony. Shown to the right and below are vertical sections through the biofilm collected at the positions indicated by the white triangles. (B) Magnification of a *P. putida* colony with green-fluorescent cells covering the surface. Vertical sections through the colony are shown to the right and below. The microcolony is a SFP of a region 10 to 19 μm from the glass surface.

transconjugants) determined by plating on the selective plates, strongly indicating that all the transconjugants carrying the TOL plasmid are also green fluorescent.

In conclusion, we have shown that a new biochemical pathway offering a growth-selective advantage can be introduced effectively in a mixed microbial surface community when carried on a conjugative plasmid. Successful establishment of the new genetic information was independent of the efficiency with which the donor strain itself could be established; however, proliferation of the transconjugant cells occurred primarily on the surfaces of preexisting colonies of isogenic recipient cells in the community.

ACKNOWLEDGMENTS

This work was supported by grants from the Danish Biotechnology Program.

Brendan Cormack, Rafael Valdivia, and Stanley Falkow are acknowledged for the gift of the *gfp*mut3b allele. We also thank Anne Nielsen and Tove Johansen for technical assistance.

REFERENCES

1. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172:762–770.
2. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143–169.
3. **Andersen, J. B., C. Sternberg, L. K. Poulsen, S. P. Bjørn, M. Givskov, and S. Molin.** 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl. Environ. Microbiol. 64:2240–2246.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41:459.
5. **Bradley, D. E., and P. A. Williams.** 1982. The TOL plasmid is naturally derepressed for transfer. J. Gen. Microbiol. 128:3019–3024.
6. **Bujard, H., and M. Lanzer.** 1988. Promoters largely determine the efficiency of repressor action. Proc. Natl. Acad. Sci. USA 85:8973–8977.
7. **Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher.** 1994. Green fluorescent protein as a marker for gene expression. Science 263:802–805.
8. **Christensen, B. B., C. Sternberg, and S. Molin.** 1996. Bacterial plasmid conjugation on semi-solid surfaces monitored with the green fluorescent protein (Gfp) from *Aequorea victoria* as a marker. Gene 173:59–65.
9. **Clark, J. D., and O. Maaløe.** 1967. DNA replication and the division cycle in *Escherichia coli*. J. Mol. Biol. 21:99–112.
10. **Cormack, B. P., R. H. Valdivia, and S. Falkow.** 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173:33–38.
11. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. 172:6568–6572.
12. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5- and Tn*10*-derived minitransposons. Methods Enzymol. 235:386–405.
13. **De Rore, H., K. Demolder, K. De Wilde, E. Top, F. Houwen, and W. Verstraete.** 1994. Transfer of the catabolic plasmid RP4::Tn4371 to indigenous soil bacteria and its effect on respiration and biphenyl breakdown. FEMS Microbiol. Ecol. 15:71–78.
14. **Dhandayuthapani, S., L. E. Via, C. A. Thomas, P. M. Horowitz, D. Deretic, and V. Deretic.** 1995. Green fluorescent protein as a marker for gene expression and cell biology of mycobacterial interactions with macrophages. Mol. Microbiol. 17:901–912.
15. **DiGiovanni, G. D., J. W. Neilson, I. L. Pepper, and N. A. Sinclair.** 1996. Gene transfer of *Alcaligenes eutrophus* JMP134 plasmid pJP4 to indigenous soil recipients. Appl. Environ. Microbiol. 62:2521–2526.
16. **Eberl, L., R. Schulze, A. Ammendola, O. Geisenberger, R. Erhart, C. Sternberg, S. Molin, and R. I. Amann.** 1997. Use of green fluorescent protein as a marker for ecological studies of activated sludge communities. FEMS Microbiol. Ecol. 149:77–83.
17. **Fulthorpe, R. R., and R. C. Wyndham.** 1989. Survival and activity of a 3-chlorobenzoate-catabolic genotype in a natural system. Appl. Environ. Microbiol. 55:1584–1590.
18. **Fulthorpe, R. R., and R. C. Wyndham.** 1991. Transfer and expression of the catabolic plasmid pBRC60 in wild bacterial recipients in a freshwater ecosystem. Appl. Environ. Microbiol. 57:1546–1553.
19. **Fulthorpe, R. R., and R. C. Wyndham.** 1992. Involvement of a chlorobenzoate-catabolic transposon, Tn*5271*, in community adaptation to chlorobiphenyl, chloroaniline and 2,4-dichlorophenoxyacetic acid in a freshwater ecosystem. Appl. Environ. Microbiol. 58:314–325.
20. **Goldstien, R. M., L. M. Mallory, and M. Alexander.** 1985. Reasons for possible failure of inoculation to enhance biodegradation. Appl. Environ. Microbiol. 50:977–983.

21. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. 172:6557–6567.
22. **Kessler, B., V. de Lorenzo, and K. N. Timmis.** 1992. A general system to integrate *lacZ* fusions into the chromosomes of Gram-negative eubacteria: regulation of the *Pm* promoter of the TOL plasmid studied with all controlling elements in monocopy. Mol. Gen. Genet. 233:293–301.
23. **Kristensen, C. S., L. Eberl, J. M. Sanchez-Romero, M. Givskov, S. Molin, and V. de Lorenzo.** 1995. Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. J. Bacteriol. 177:52–58.
24. **Lawrence, J. R., D. R. Korber, G. M. Wolfaardt, and D. E. Caldwell.** 1995. Bacterial behavioural strategies at interfaces, p. 1–75. *In* J. G. Jones (ed.), Advances in microbial ecology. Plenum Press, New York, N.Y.
25. **Leser, T. D., M. Boye, and N. B. Hendriksen.** 1995. Survival and activity of *Pseudomonas* sp. strain B13(FR1) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and its effect on the indigenous bacterioplankton. Appl. Environ. Microbiol. 61:1201–1207.
26. **Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese.** 1997. The RDP (Ribosomal Database Project). Nucleic Acids Res. 25:109–110.
27. **Massol-Deyá, A. A., J. Whallon, R. F. Hickey, and J. M. Tiedje.** 1995. Channel structures in aerobic biofilms of fixed-film reactors treating contaminated groundwater. Appl. Environ. Microbiol. 61:769–777.
28. **McClure, N. C., A. J. Weightman, and J. C. Fry.** 1989. Survival of *Pseudomonas putida* UWC1 containing cloned catabolic genes in a model activated-sludge unit. Appl. Environ. Microbiol. 55:2627–2634.
29. **Møller, S., A. R. Pedersen, L. K. Poulsen, E. Arvin, and S. Molin.** 1996. Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative in situ hybridization and scanning confocal laser microscopy. Appl. Environ. Microbiol. 62:4632–4640.
30. **Møller, S., C. Sternberg, J. B. Andersen, B. B. Christensen, J. L. Ramos, M. Givskov, and S. Molin.** 1998. In situ gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. Appl. Environ. Microbiol. 64:721–732.
31. **Nielson, J. W., K. L. Josephson, S. D. Pillai, and I. L. Pepper.** 1992. Polymerase chain reaction and gene probe detection of the 2,4-dichlorophenoxyacetic acid degradation plasmid, pJP4. Appl. Environ. Microbiol. 58:1271–1275.
32. **Nüblein, K., D. Maris, K. Timmis, and D. F. Dwyer.** 1992. Expression and transfer of engineered catabolic pathways harbored by *Pseudomonas* spp. introduced into activated sludge microcosms. Appl. Environ. Microbiol. 58:3380–3386.
33. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. 59:1354–1360.
34. **Sayler, G. S., and A. C. Layton.** 1990. Environmental application of nucleic acid hybridization. Annu. Rev. Microbiol. 44:625–648.
35. **Shapiro, J. A.** 1984. The use of Mud*lac* transposons as tools for vital staining to visualize clonal and non-clonal patterns of organization in bacterial growth on agar surfaces. J. Gen. Microbiol. 130:1169–1181.
36. **Smets, B. F., B. E. Rittmann, and D. A. Stahl.** 1993. The specific growth rate of *Pseudomonas putida* PAW1 influences the conjugal transfer rate of the TOL plasmid. Appl. Environ. Microbiol. 59:3430–3437.
37. **Smets, B. F., B. E. Rittmann, and D. A. Stahl.** 1995. Quantification of the effect of substrate concentration on the conjugal transfer rate of the TOL plasmid in short-term batch mating experiments. Lett. Appl. Microbiol. 21:167–172.
38. **Stark, M. J. R.** 1987. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression. Gene 51:255–267.
39. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. 54:2185–2191.
40. **Veal, D. A., H. W. Stokes, and G. Daggard.** 1992. Genetic exchange in natural microbial communities. Adv. Microb. Ecol. 12:383–430.
41. **Wagner, M., R. I. Amann, P. Kämpfer, B. Assmus, A. Hartmann, P. Hutzler, N. Springer, and K. H. Schleifer.** 1995. Identification and *in situ* detection of gram-negative filamentous bacteria in activated sludge. Syst. Appl. Microbiol. 17:405–417.
42. **Webb, C. D., A. Decatur, A. Teleman, and R. Losick.** 1995. Use of green fluorescent protein for visualization of cell-specific gene expression and subcellular protein localization during sporulation in *Bacillus subtilis*. J. Bacteriol. 177:5906–5911.
43. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. 120:416–423.
44. **Winkler, J., K. N. Timmis, and R. A. Snyder.** 1995. Tracking the response of *Burkholderia cepacia* G4 5223-PR1 in aquifer microcosms. Appl. Environ. Microbiol. 61:448–455.
45. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.
46. **Wolfaardt, G. M., J. R. Lawrence, J. V. Headly, R. D. Robarts, and D. E. Caldwell.** 1994. Microbial exopolymers provide a mechanism for bioaccumulation of contaminants. Microb. Ecol. 27:279–291.
47. **Wolfaardt, G. M., J. R. Lawrence, R. D. Robarts, S. J. Caldwell, and D. E. Caldwell.** 1994. Multicellular organization in a degradative biofilm community. Appl. Environ. Microbiol. 60:434–446.
48. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas* (*putida* (*arvilla*) [sic] mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. 124:7–13.
49. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119.